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I, JULIE BILLINGSLEY, TEAM LEADER EXAMINATION SUPPORT AND SALES hereby certify that annexed is a true copy of the Provisional specification in connection with Application No. 2003901876 for a patent by THE MACFARLANE BURNET INSTITUTE FOR MEDICAL RESEARCH AND PUBLIC HEALTH as filed on 17 April 2003.

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J. Billingsley

JULIE BILLINGSLEY
TEAM LEADER EXAMINATION
SUPPORT AND SALES

Regulation 3.2

AUSTRALIA

Patents Act 1990

PROVISIONAL SPECIFICATION

for the invention entitled:

"Viral Vector"

The invention is described in the following statement:

VIRAL VECTORS

BACKGROUND OF THE INVENTION

FIELD OF THE INVENTION

The present invention relates generally to virus-like particles (VLPs) for use in delivering a polypeptide of interest to a subject and to nucleic acid molecules encoding same. In particular, the present invention takes advantage of features of avian hepadnavirus particles to generate stable recombinant VLPs which are useful in the delivery to a subject of a polypeptide of interest. The polypeptide of interest may comprise one or more antigens capable of eliciting an immune response. The VLPs comprising the polypeptide of interest are useful for delivery of an antigen to the immune system of a subject. The present invention extends, *inter alia,* to plasmids, cells, kits and methods which are useful in the generation, production, delivery and monitoring of the instant VLPs.

DESCRIPTION OF THE PRIOR ART

Bibliographic details of the publications referred to by author in this specification are collected at the end of the description.

Reference herein to prior art, including any one or more prior art documents, is not to be taken as an acknowledgment, or suggestion, that said prior art is part of the state of the art.

The hepadnaviruses are a family of enveloped DNA viruses. Assembly of mammalian hepadnaviruses, such as hepatitis B virus, is complex and mature virions are formed by the interaction of preformed cytoplasmic core particles with pre-assembled surface proteins on the host endoplasmic reticulum (ER) membrane. Following interaction with appropriate portions of envelope proteins, the nucleocapsids bud into the lumen of the ER along with a 1000-fold excess of empty, subviral particles (SVPs) and assembly is completed in an

intermediate, pre-Golgi compartment (as reviewed by Nassal, M., Curr. Top. Microbiol. Immunol. *214*:297-337, 1996).

In many studies, virus-like particles (VLPs), which lack nucleocapsids, have proven to be promising candidate vaccines since they: (i) are non-infectious and therefore safe to produce and use, (ii) are more immunogenic than subunit vaccines because they provide the necessary spatial structure for display of epitopes, and (iii) elicit humoral, cell-mediated and importantly, mucosal immunity (Krueger, *et al.*, Biol. Chem. *380*:275-276,1999).

A recent example of a successful VLP vaccine, currently in clinical trials, is the recombinant papillomavirus major capsid protein (L1) VLP, which prevents infection by inducing a strong neutralizing antibody response (Frazer, Virus Research, *89*:271-274, 2002).

The hepatitis B virus (HBV) subviral particle (HBsAg-S) has been viewed as a candidate to produce recombinant VLPs. Several studies have examined which domains are suitable for insertion of foreign epitopes (Bruss *et al.*, J. Virol. *65*:3813-3820, 1994; Delpeyroux *et al J.* Mol. Biol. *195*:343-350,1987), including N terminal fusion of the preS domain (Prange, *et al*, J. Gen. Virol. *76*:2131- 2140, 1995).

Most recently, particles carrying small, 35 amino acid insertions of the hepatitis C virus (HCV) hypervariable region 1 of the E2 envelope protein into the exposed 'a' determinant in the second hydrophilic loop have successfully elicited antibody responses (Netter, *et al*, J. Virol. *75*:2130-2141, 2001). Notably, there have been limitations to the size of the inserts tolerated for particle stability and a loss of immune reactivity to the 'a' determinant of HBsAg when particles were produced in a mammalian cell system (Prange *et al*, 1995, *supra*; Bruss *et al*, J. Virol. *65*:3813-3820, 1991).

Particle instability with large fusions has recently been overcome with a Dengue virus/HBsAg fusion by expression in yeast (Bisht, *et al*, J. Biotechnology. *99*:97-110, 2002).

However, in all these cases, in order to assemble chimeric particles, the recombinant S protein must assemble with wild type S subunits. These extended S chains present a difficulty for inclusion in the tight envelope lattice formed by the HBsAg (which excludes L) and so their number is limited, and consequently the immune response generated to the foreign epitopes is low.

Accordingly, there is a need for improved VLPs which efficiently incorporate polypeptides of interest.

SUMMARY OF THE INVENTION

Throughout this specification and the claims which follow, unless the context requires otherwise, the word "comprise", and variations such as "comprises" and "comprising", will be understood to imply the inclusion of a stated integer or step or group of integers or steps but not the exclusion of any other integer or step or group of integers or steps.

Nucleotide sequences are referred to by sequence identifier numbers (SEQ ID NO:). The SEQ ID NOs: correspond numerically to the sequence identifiers <400>1, <400>2, etc.

The envelope protein of duck hepatitis B virus (DHBV) consists of two proteins, the large envelope protein (L) and the small envelope protein (S), which are produced by differential in-frame translation initiation from a single preS/S open reading frame. L and S polypeptides have a common C terminal membrane spanning or S domain, while L has an approximately 160 amino acid N-terminal extension (or preS domain) encompassing a receptor binding region. The S polypeptide is the major viral envelope constituent, which determines envelope curvature and can drive particle secretion even in the absence of the nucleocapsid. In contrast L polypeptide can only be exported when co-assembled with S.

The assembly of DHBV envelope proteins and their involvement in host cell entry are closely linked to a unique topological switch adopted by hepadnaviruses, in which a large N-terminal preS domain of the L protein is post-translocationally translocated across the ER membrane. This process is regulated so that generally only approximately 50% of molecules have translocated N-termini and the mature particle contains mixed internal/external topologies, including a partially translocated or intermediate form.

The present invention is predicted in part on the surprising discovery that substantial regions of L polypeptide of DHBV are dispensable for L translocation and particle assembly, including regions in the S domain which have the same amino acid sequence as S polypeptide regions essential for particle assembly. Accordingly, L polypeptides are

more flexible in their particle association than S polypeptides and thus open to more extensive manipulation.

The present invention therefore provides virus like particles (VLPs) which consist primarily of a small envelope (S) polypeptide or a functional derivative or homolog thereof. In addition, however, they comprise a fusion polypeptide comprising a polypeptide of interest (POI) and at least a particle associating portion of a large envelope (L) polypeptide or a functional derivative or homolog thereof. Because the L polypeptide is not excluded during VLP assembly and because it can be extensively manipulated to vector a heterologous polypeptide without significantly affecting particle stability, the VLP of the present invention will be useful, *inter alia*, in the delivery of a polypeptide of interest to a subject.

The present invention is described with particular reference to DHBV, however, the invention extends to L and S polypeptides from other viruses with L and S envelope polypeptides proteins in which L is not excluded from particle assembly. For example, L and S polypeptides from other avian hepadnaviruses are contemplated such as, but not limited to such heron (HHBV), snow goose (SGHBV) and hepadnaviruses which exhibit similar subviral particle morphology to DHBV, i.e., with L and S envelope proteins. The S domains of L and S polypeptides are highly conserved within all hepadnaviruses, exhibiting for example up to 70% amino acid similarity in the region between TM1 and TM2.

The present invention furthermore provides, a virus-like particle (VLP) comprising i) a fusion polypeptide comprising a polypeptide of interest (POI) and a particle associating portion of a large envelope (L) polypeptide of DHBV or a functional derivative or homolog thereof, and ii) a small envelope (S) polypeptide of DHBV or a functional derivative or homolog thereof.

By introducing one or more POIs into the L polypeptide, the POI is translocated along with L into a particle structure made up primarily of S polypeptide.

Although several examples of particle associating portions of the L polypeptide are provided, based on the teaching provided herein it would be well within the skill of the addressee to generate smaller or larger portions of L-polypeptide and determine whether they, together with a heterologous protein of interest, are capable of particle association within a VLP. Accordingly, the present invention is in no way limited to the exemplified constructs.

The virus like particles of the present invention are useful in vaccine compositions to promote an effective immune response. In particular embodiments, the virus-like particles are advantageously a suitable size to be taken up by antigen presenting cells, such as dendritic cells. For example, DHBV VLPs are significantly larger than mammalian HBV VLPs.

Recombinant VLPs of the present invention are prepared using expression plasmids comprising nucleic acid molecules encoding the recombinant L polypeptides separately or together with S polypeptides. Expression plasmids are conveniently in a form suitable for expression in yeast, baculovirus or mammalian expression systems. Plasmids comprising nucleic acid molecules encoding the instant recombinant L polypeptides may also be used to deliver nucleic acid constructs directly to the cells of a subject. Alternatively, kits are contemplated to facilitate recombinant production of the instant fusion proteins.

The present invention furthermore provides methods for producing a recombinant virus-like particle and compositions comprising a virus-like particle derived from L and S polypeptide of avian hepadnaviruses or other non-L excluding viruses for use as a vaccine wherein the L polypeptide comprises one or more antigens of interest derived from one or more heterologous sources.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 is a schematic representation of a cloning strategy for generating $_p$CDL-E2.

Figure 2A is a schematic representation of the large (L) and small (S) envelope proteins of DHBV. L and S are produced by differential translation from a single open reading frame such that L protein consists of a preS domain of 161 amino acids and a C-terminal S domain of 167 amino acids, which comprises the S protein. The three transmembrane domains (TM) are indicated by the boxes.

Figure 2B is a schematic representation of L showing where the 82 amino acid portion of the HCV E2 ectodomain was inserted into the preS domain, generating the E2/L chimeric envelope protein.

Figure 2C provides the results of a Western Blot showing that the E2/L chimera is translocated across the ER. Protease protection analysis of ER microsomes prepared from LMH cells transfected with pCDL-E2 and pCI-S (an S protein expression plasmid). Microsomes samples were subjected to digestion with trypsin in the absence or presence of the detergent, NP-40, or left untreated, as denoted above each lane. Protease protection of E2/L chains was analysed by SDS-PAGE and Western blotting with a monoclonal anti-S antibody, which detects both E2/L and S proteins. Protection of E2/L from trypsin digestion (middle lane) is an indication of translocation to the ER lumen.

Figure 2D provides the results of a Western Blot showing that the E2/L chimera is assembled into particles. Intracellular particles were isolated from avian hepatoma (LMH) cells transfected with pCDL-E2 and pCI-S by freeze-thawing cells 3 times, centrifugation to obtain the cytosolic fraction for sedimentation of particles through 20% sucrose on to a 70% sucrose cushion at 38,000 r.p.m. (SW41 rotor Beckman). The particle fraction at the 20-70% sucrose interface was methanol precipitated prior to SDS-PAGE and analysis of envelope proteins by Western blotting.

Figure 2E is a schematic representation modelling the membrane orientations of L protein on the ER (depicted as microsome vesicles), showing the process of post-translational translocation to the microsome lumen, which confers protection from trypsin digestion of the E2/L hybrid chains. During particle assembly assembled envelope proteins bud from the ER into the ER lumen taking the inner leaflet of the ER membrane. Particles are exported from the cell via the cellular vesicular export pathway enabling isolation of particles both from the cytosolic (as shown in D) and extracellular compartments. Envelope protein domains translocated to the ER lumen are thus ultimately exposed to the outside of the assembled particle, as indicated by the schematic diagram of a particle.

Figure 3 provides the genomic nucleotide sequence of DHBV.

Figure 4 provides the amino acid sequences of L and S polypeptides of DHBV. Start sites are underlined and stop sites are starred (*).

TABLE 1

SEQ ID NO.	SEQUENCE
SEQ ID NO: 1	Primers for generating L-fusion proteins
SEQ ID NO: 2	Primers for generating L-fusion proteins
SEQ ID NO: 3	Primers for generating L-fusion proteins
SEQ ID NO: 4	Primers for generating L-fusion proteins
SEQ ID NO: 5	Full genomic nucleotide sequence of DHBV
SEQ ID NO: 6	Nucleotide sequence of L polypeptide of DHBV
SEQ ID NO: 7	Amino acid sequence of L polypeptide of DHBV
SEQ ID NO: 8	Nucleotide sequence of S domain of L polypeptide of DHBV
SEQ ID NO: 9	Amino acid sequence of S domain of L polypeptide of DHBV
SEQ ID NO: 10	Nucleotide sequence of preS domain of L polypeptide of DHBV
SEQ ID NO: 11	Amino acid sequence of pre S domain of L polypeptide of DHBV
SEQ ID NO: 12	Nucleotide sequence of S polypeptide of DHBV
SEQ ID NO: 13	Amino acid sequence of S polypeptide of DHBV

DETAILED DESCRIPTION OF THE INVENTION

The present invention is predicated in part on the finding that L polypeptide of DHBV can be substantially modified yet still retain the ability to associate with S polypeptides of DHBV during virus-like particle assembly.

It is proposed herein to use an L polypeptide or a functional derivative or homolog thereof to deliver a polypeptide of interest to a subject. In particular, it is proposed to use an L polypeptide to deliver antigens of interest to the immune system of a subject as part of a virus-like particle.

In a broad embodiment the present invention provides a virus-like particle (VLP) comprising a fusion polypeptide comprising a polypeptide of interest (POI) and a particle associating portion of a large envelope (L) polypeptide.

The virus-like particle is primarily composed of a small envelope (S) polypeptide.

Accordingly, the present invention furthermore provides a VLP comprising i) fusion polypeptide comprising a polypeptide of interest (POI) and a particle associating portion of a large envelope (L) polypeptide or a functional derivative or homolog thereof, and ii) a small envelope (S) polypeptide of DHBV or a functional derivative or homolog thereof.

More specifically, the present invention furthermore provides a VLP comprising i) a fusion polypeptide comprising a polypeptide of interest (POI) and a particle associating portion of a large envelope (L) polypeptide of DHBV or a functional derivative or homolog thereof, and ii) a small envelope (S) polypeptide of DHBV or a functional derivative or homolog thereof.

Preferably at least part of said POI is exposed on the surface of the virus-like particle.

The term "virus-like particle" is used in its broadest sense to mean a particle or three dimensional proteinaceous structure which, like sub-viral particles of enveloped viruses, form particles by self-assembly or folding of envelope polypeptides within a lipid bilayer. The virus-like particles of the present invention may be recombinant or synthetic or may comprise a combination of synthetic and recombinant components.

Reference herein to the singular form such as "a", "an" or "the" includes the plural aspect unless the context clearly specifies otherwise. Thus, reference for example to "a polypeptide of interest" includes a single polypeptide, as well as two or more such polypeptides.

Reference herein to the term "polypeptide" means a polymer of amino acids and should not be limited to any particular length. Therefore, the term includes proteins, oligopeptides, peptides and epitopes. The term does not exclude modifications of the polypeptide, for example myristylation, glycosylation, phosphorylation, addition of N-terminal signal sequences and the like. Included within the definition are, for example, polypeptides containing one or more analogs of an amino acid (including for example, unnatural amino acids such as those give in Table 2) or polypeptides with substituted linkages.

Analogs contemplated herein include but are not limited to modification to side chains, incorporating of unnatural amino acids and/or their derivatives during peptide, polypeptide or protein synthesis and the use of crosslinkers and other methods which impose conformational constraints on the proteinaceous molecule or their analogs.

Examples of side chain modifications contemplated by the present invention include modifications of amino groups such as by reductive alkylation by reaction with an aldehyde followed by reduction with $NaBH_4$; amidination with methylacetimidate; acylation with acetic anhydride; carbamoylation of amino groups with cyanate; trinitrobenzylation of amino groups with 2, 4, 6-trinitrobenzene sulphonic acid (TNBS); acylation of amino groups with succinic anhydride and tetrahydrophthalic anhydride; and pyridoxylation of lysine with pyridoxal-5-phosphate followed by reduction with $NaBH_4$.

The guanidine group of arginine residues may be modified by the formation of heterocyclic condensation products with reagents such as 2,3-butanedione, phenylglyoxal and glyoxal.

The carboxyl group may be modified by carbodiimide activation *via* O-acylisourea formation followed by subsequent derivitization, for example, to a corresponding amide.

Sulphydryl groups may be modified by methods such as carboxymethylation with iodoacetic acid or iodoacetamide; performic acid oxidation to cysteic acid; formation of a mixed disulphides with other thiol compounds; reaction with maleimide, maleic anhydride or other substituted maleimide; formation of mercurial derivatives using 4-chloromercuribenzoate, 4-chloromercuriphenylsulphonic acid, phenylmercury chloride, 2-chloromercuri-4-nitrophenol and other mercurials; carbamoylation with cyanate at alkaline pH, palmitylation of cysteine residues.

Tryptophan residues may be modified by, for example, oxidation with N-bromosuccinimide or alkylation of the indole ring with 2-hydroxy-5-nitrobenzyl bromide or sulphenyl halides. Tyrosine residues on the other hand, may be altered by nitration with tetranitromethane to form a 3-nitrotyrosine derivative.

Modification of the imidazole ring of a histidine residue may be accomplished by alkylation with iodoacetic acid derivatives or N-carbethoxylation with diethylpyrocarbonate.

Examples of incorporating unnatural amino acids and derivatives during peptide synthesis include, but are not limited to, use of norleucine, 4-amino butyric acid, 4-amino-3-hydroxy-5-phenylpentanoic acid, 6-aminohexanoic acid, t-butylglycine, norvaline, phenylglycine, ornithine, sarcosine, 4-amino-3-hydroxy-6-methylheptanoic acid, 2-thienyl alanine and/or D-isomers of amino acids. A list of unnatural amino acid, contemplated herein is shown in Table 2.

TABLE 2

Codes for non-conventional amino acids

Non-conventional amino acid	Code	Non-conventional amino acid	Code
α-aminobutyric acid	Abu	L-N-methylalanine	Nmala
α-amino-α-methylbutyrate	Mgabu	L-N-methylarginine	Nmarg
aminocyclopropane-carboxylate	Cpro	L-N-methylasparagine	Nmasn
		L-N-methylaspartic acid	Nmasp
aminoisobutyric acid	Aib	L-N-methylcysteine	Nmcys
aminonorbornyl-carboxylate	Norb	L-N-methylglutamine	Nmgln
		L-N-methylglutamic acid	Nmglu
cyclohexylalanine	Chexa	L-Nmethylhistidine	Nmhis
cyclopentylalanine	Cpen	L-N-methylisolleucine	Nmile
D-alanine	Dal	L-N-methylleucine	Nmleu
D-arginine	Darg	L-N-methyllysine	Nmlys
D-aspartic acid	Dasp	L-N-methylmethionine	Nmmet
D-cysteine	Dcys	L-N-methylnorleucine	Nmnle
D-glutamine	Dgln	L-N-methylnorvaline	Nmnva
D-glutamic acid	Dglu	L-N-methylornithine	Nmorn
D-histidine	Dhis	L-N-methylphenylalanine	Nmphe
D-isoleucine	Dile	L-N-methylproline	Nmpro
D-leucine	Dleu	L-N-methylserine	Nmser
D-lysine	Dlys	L-N-methylthreonine	Nmthr
D-methionine	Dmet	L-N-methyltryptophan	Nmtrp
D-ornithine	Dorn	L-N-methyltyrosine	Nmtyr
D-phenylalanine	Dphe	L-N-methylvaline	Nmval
D-proline	Dpro	L-N-methylethylglycine	Nmetg
D-serine	Dser	L-N-methyl-t-butylglycine	Nmtbug

	D-threonine	Dthr	L-norleucine	Nle
	D-tryptophan	Dtrp	L-norvaline	Nva
	D-tyrosine	Dtyr	α-methyl-aminoisobutyrate	Maib
	D-valine	Dval	α-methyl-γ-aminobutyrate	Mgabu
5	D-α-methylalanine	Dmala	α-methylcyclohexylalanine	Mchexa
	D-α-methylarginine	Dmarg	α-methylcylcopentylalanine	Mcpen
	D-α-methylasparagine	Dmasn	α-methyl-α-napthylalanine	Manap
	D-α-methylaspartate	Dmasp	α-methylpenicillamine	Mpen
	D-α-methylcysteine	Dmcys	N-(4-aminobutyl)glycine	Nglu
10	D-α-methylglutamine	Dmgln	N-(2-aminoethyl)glycine	Naeg
	D-α-methylhistidine	Dmhis	N-(3-aminopropyl)glycine	Norn
	D-α-methylisoleucine	Dmile	N-amino-α-methylbutyrate	Nmaabu
	D-α-methylleucine	Dmleu	α-napthylalanine	Anap
	D-α-methyllysine	Dmlys	N-benzylglycine	Nphe
15	D-α-methylmethionine	Dmmet	N-(2-carbamylethyl)glycine	Ngln
	D-α-methylornithine	Dmorn	N-(carbamylmethyl)glycine	Nasn
	D-α-methylphenylalanine	Dmphe	N-(2-carboxyethyl)glycine	Nglu
	D-α-methylproline	Dmpro	N-(carboxymethyl)glycine	Nasp
	D-α-methylserine	Dmser	N-cyclobutylglycine	Ncbut
20	D-α-methylthreonine	Dmthr	N-cycloheptylglycine	Nchep
	D-α-methyltryptophan	Dmtrp	N-cyclohexylglycine	Nchex
	D-α-methyltyrosine	Dmty	N-cyclodecylglycine	Ncdec
	D-α-methylvaline	Dmval	N-cylcododecylglycine	Ncdod
	D-N-methylalanine	Dnmala	N-cyclooctylglycine	Ncoct
25	D-N-methylarginine	Dnmarg	N-cyclopropylglycine	Ncpro
	D-N-methylasparagine	Dnmasn	N-cycloundecylglycine	Ncund
	D-N-methylaspartate	Dnmasp	N-(2,2-diphenylethyl)glycine	Nbhm
	D-N-methylcysteine	Dnmcys	N-(3,3-diphenylpropyl)glycine	Nbhe
	D-N-methylglutamine	Dnmgln	N-(3-guanidinopropyl)glycine	Narg
30	D-N-methylglutamate	Dnmglu	N-(1-hydroxyethyl)glycine	Nthr

	D-N-methylhistidine	Dnmhis	N-(hydroxyethyl))glycine	Nser
	D-N-methylisoleucine	Dnmile	N-(imidazolylethyl))glycine	Nhis
	D-N-methylleucine	Dnmleu	N-(3-indolylyethyl)glycine	Nhtrp
	D-N-methyllysine	Dnmlys	N-methyl-γ-aminobutyrate	Nmgabu
5	N-methylcyclohexylalanine	Nmchexa	D-N-methylmethionine	Dnmmet
	D-N-methylornithine	Dnmorn	N-methylcyclopentylalanine	Nmcpen
	N-methylglycine	Nala	D-N-methylphenylalanine	Dnmphe
	N-methylaminoisobutyrate	Nmaib	D-N-methylproline	Dnmpro
	N-(1-methylpropyl)glycine	Nile	D-N-methylserine	Dnmser
10	N-(2-methylpropyl)glycine	Nleu	D-N-methylthreonine	Dnmthr
	D-N-methyltryptophan	Dnmtrp	N-(1-methylethyl)glycine	Nval
	D-N-methyltyrosine	Dnmtyr	N-methyla-napthylalanine	Nmanap
	D-N-methylvaline	Dnmval	N-methylpenicillamine	Nmpen
	γ-aminobutyric acid	Gabu	N-(*p*-hydroxyphenyl)glycine	Nhtyr
15	L-*t*-butylglycine	Tbug	N-(thiomethyl)glycine	Ncys
	L-ethylglycine	Etg	penicillamine	Pen
	L-homophenylalanine	Hphe	L-α-methylalanine	Mala
	L-α-methylarginine	Marg	L-α-methylasparagine	Masn
	L-α-methylaspartate	Masp	L-α-methyl-*t*-butylglycine	Mtbug
20	L-α-methylcysteine	Mcys	L-methylethylglycine	Metg
	L-α-methylglutamine	Mgln	L-α-methylglutamate	Mglu
	L-α-methylhistidine	Mhis	L-α-methylhomophenylalanine	Mhphe
	L-α-methylisoleucine	Mile	N-(2-methylthioethyl)glycine	Nmet
	L-α-methylleucine	Mleu	L-α-methyllysine	Mlys
25	L-α-methylmethionine	Mmet	L-α-methylnorleucine	Mnle
	L-α-methylnorvaline	Mnva	L-α-methylornithine	Morn
	L-α-methylphenylalanine	Mphe	L-α-methylproline	Mpro
	L-α-methylserine	Mser	L-α-methylthreonine	Mthr
	L-α-methyltryptophan	Mtrp	L-α-methyltyrosine	Mtyr
30	L-α-methylvaline	Mval	L-N-methylhomophenylalanine	Nmhphe

N-(N-(2,2-diphenylethyl) carbamylmethyl)glycine	Nnbhm	N-(N-(3,3-diphenylpropyl) carbamylmethyl)glycine	Nnbhe
1-carboxy-1-(2,2-diphenyl-ethylamino)cyclopropane	Nmbc		

Crosslinkers can be used, for example, to stabilize 3D conformations, using homo-bifunctional crosslinkers such as the bifunctional imido esters having $(CH_2)_n$ spacer groups with n=1 to n=6, glutaraldehyde, N-hydroxysuccinimide esters and hetero-bifunctional reagents which usually contain an amino-reactive moiety such as N-hydroxysuccinimide and another group specific-reactive moiety such as maleimido or dithio moiety (SH) or carbodiimide (COOH). In addition, peptides can be conformationally constrained by, for example, incorporation of C_α and N $_\alpha$-methylamino acids and the introduction of double bonds between C_α and C_β atoms of amino acids.

The terms "fusion polypeptide" or "chimeric polypeptide" or "hybrid polypeptide " are interchangably used to mean a polypeptide comprising two or more associated polypeptides which are expressed as part of the same expression product, or which are generated by synthetic means. Fusion polypeptides may comprise two or more L and POI polypeptides and intervening regions such as, for example, linker or spacer regions. In particular, regions which permit or directly or indirectly facilitate a surface topology or increase protease resistance for the polypeptide of interest in the particle are contemplated, for example, N-terminal signal sequences. An example of a spacer region is a transmembrane domain. Alternatively, or in addition, regions which promote a cytosolic topology may be included. Polypeptide topology in a viral particle may be assessed for example by protease protection assay or by determining interactivity with antibodies determined by the L polypeptide, S polypeptide, the polypeptide of interest or epitopes generated through fusion of these polypeptides. According, the term "fusion" in "fusion polypeptide" is not used in the sense of "viral fusion".

The term "polypeptide of interest" means any polypeptide which is advantageously delivered to a subject as part of a virus-like particle. For example, two or more or a matrix of polypeptides involved in promoting and/or mediating a particular biochemical or physiological reaction may be delivered to a subject in viral particle form. A particular reaction contemplated is an immune response to an antigen. Accordingly the term includes any antigenic polypeptide of interest. Antigenic polypeptides may be co-expressed with immunopotentiating polypeptides such as cytokines as is well known in the art.

"Subject" as used herein refers to an animal, preferably a mammal and more preferably human who can benefit from administration of the viral particles of the present invention. There is no limitation on the type of animal that could benefit from the presently described molecules. A patient regardless of whether a human or non-human animal may be referred to as an individual, subject, animal, host or recipient. The molecules and methods of the present invention have applications in human medicine, veterinary medicine as well as in general, domestic or wild animal husbandry. For convenience, an "animal" includes an avian species such as a poultry bird, an aviary bird or game bird.

The preferred animals are humans or other primates, livestock animals, laboratory test animals, companion animals or captive wild animals.

Examples of laboratory test animals include ducks, snow geese, mice, rats, rabbits, guinea pigs and hamsters. Rabbits and rodent animals, such as rats and mice, provide a convenient test system or animal model. Livestock animals include sheep, cows, pigs, goats, horses and donkeys. Non-mammalian animals such as avian species, zebrafish and amphibians are also contemplated.

The terms "antigen" or "antigenic polypeptide" are used in their broadest sense to include polypeptides which are capable of inducing an immune response in a subject. The antigenic polypeptide may comprise single epitope regions through to multiple epitope regions including repeated epitope regions. The antigenic polypeptide may derive from a single or multiple sources although antigens from infectious agents, such as, for example,

viruses, bacteria, fungi, protozoa, trematodes, nematodes, prions and the like are contemplated, as are tumour-related antigens. Antigenic regions of many agents and tumour-related proteins are well known in the art.

As is well know to those skilled in the art, effective immune responses for prophylactic or therapeutic vaccines generally elicit strong CTL and T-helper cell responses as well as strong humoral responses.

The antigenic polypeptide of interest may comprise epitope regions from two or more polypeptides from different organisms, species or subspecies. For example, viral and bacterial or multiple viral or multiple bacterial infectious may be vaccinated for simultaneously.

The phrase "particle associating portion" means for all L polypeptides, that or those portions of the L polypeptide which is/are required for L polypeptide incorporation into virus-like particles. For example, the TM1 region of the S domain of L is not required for L association with the particle and may be omitted from the L-polypeptide used herein. Indeed, as contemplated herein, the TM2 and downstream sequence of L polypeptide are sufficient for particle association. Similarly, the preS domain of L is not required for assembly of L in the particles. The S domain of L absent TM1 is an example of a particle associating portion of L. Many different particle association portions are clearly available pursuant to the present invention. The nature of this portion is flexible and may be determined empirically using methods known in the art and referred to herein.

Although a minimum functional portion of L may be advantageous in some applications, the present invention extends full length L polypeptides interspersed with a POI or wherein the POI is terminally appended. Preferably the POI is introduced into surface exposed portions of L.

The term "derived from" means that a particular element or group of elements has originated from the source described, but has not necessarily been obtained directly from the specified source.

The term "isolated" includes reference to VLPs having undergone at least one purification step, conveniently described in terms of the % of pure material in a sample.

In another aspect of the present invention a virus-like particle (VLP) is contemplated comprising i) a fusion polypeptide comprising a polypeptide of interest (POI) and at least a portion of the S domain of a large envelope (L) polypeptide of DHBV or a functional derivative or homolog thereof; and ii) a small envelope (S) polypeptide of DHBV or a functional derivative or homolog thereof, wherein at least a part of said POI is exposed on the surface of the virus-like particle.

Exemplary portions are amino acids 24 to 167 of DHBV S domain or, more preferably at least TM2 and downstream sequences of L polypeptide of DHBV.

In one particular embodiment of the present invention, the polypeptide of interest is located at the amino terminal side of the S domain amino acid sequence of the L polypeptide or the S domain minus the TM1 domain. In another embodiment, the POI is located in the pre-S domain of the L polypeptide or N terminally to the L polypeptide.

By introducing one or more POIs into the pre-S domain of L or N terminally to the S domain of L or N-terminally to the S domain absent TM1, the POI is translocated along with L into a particle structure made up primarily of S polypeptide. This facilitates a high copy number of POI per VLP.

In one embodiment the virus-like particles of the present invention are useful in vaccine compositions to promote an effective immune response. In particular, the virus like particles are advantageously a suitable size to be taken up by antigen presenting cells, such as dendritic cells. Specifically, in relation to mammalian hepadnavirus particles, these are

typically approximately 20 nanometers, while those of avian hepadnaviruses are pleomorphic and are typically between 35 and 60 nanometers in diameter. An effective immune response is one which is capable of reducing the number of target antigens in a subject and may prevent infections or development of disease conditions (prophylactic vaccine) or may treat current infections or conditions (therapeutic vaccination).

Without being bound to any particular theory, the VLPs of the present invention are capable of stimulating humoral and/or cell mediated immune responses. Heterologous antigens are targeted to appropriate pathways of MHC class 1 and class 11 antigen processing and presentation, and are targeted for dendritic cells which initiate, in particular T-cell responses.

Preferred L polypeptides comprise or consist of an amino acid sequence substantially set forth in SEQ ID NO: 7 and SEQ ID NO: 9, or an amino acid sequence having at least 50% similarity to SEQ ID NO: 7 or SEQ ID NO: 9.

Preferred L polypeptides are derived from an avian hepadnavirus such as but not limited to DHBV.

Functional derivatives of the instant L polypeptide include fragments, parts or portions of the parent molecule which retain the ability of the L polypeptide to associate with the particle formed by S polypeptide, or at least where such ability is not substantially lost

Functional derivatives of the instant S polypeptide retain the ability of the ability of the S polypeptide to form virus-like particles, or at least where such ability is not substantially lost.

Substantial loss would mean that the L particle is assembled with S in particles at a ratio of less than about 1:1 or more preferably less than about 1:2, even more preferably less than about 1:3, still even more preferably less than about 1:4.

Preferred S polypeptide are derived from an avian hepadnavirus such as but not limited to DHBV or comprise or consist of an amino acid sequence substantially set forth in SEQ ID NO: 13.

The term "functional derivative" also extends to polypeptides having one or more amino acid mutations or modifications. Mutations may be derived from additions, insertions, deletions or substitutions of amino acids. Substitutions may for example be conservative amino acid substitutions within the following groups: glycine and alanine; valine, isoleucine and leucine; aspartic acid and glutamic acid; asparagine and glutamine; serine and threonine; lysine and arginine; and phenyl alanine and tyrosine. Modifications may include the addition of flanking sequences which enhance viral particle assembly or stability.

Preferably, derivatives have at least 60% amino acid similarity, or more preferably at least 80%, or most preferably 90% or greater similarity to the parent molecules.

Accordingly, in another embodiment, the present invention provides a VLP comprising a fusion polypeptide comprising a POI and a particle associating portion of an L polypeptide wherein said L polypeptide comprises a sequence of amino acids substantially as set forth in SEQ ID NO: 7 or SEQ ID NO: 9 or an amino acid sequence having at least about 50% similarity thereto, or a functional derivative or homolog thereof.

In another embodiment, the present invention provides a VLP comprising a fusion polypeptide comprising a POI and a particle associating portion of an L polypeptide wherein said L polypeptide is encoded by a sequence of nucleotides substantially as set forth in SEQ ID NO: 6 or SEQ ID NO: 8 or a sequence of nucleotides capable of hybridizing to SEQ ID NO: 6 or SEQ ID NO: 8, or a complementary form thereof under medium stringency conditions.

In a preferred embodiment, hybridization conditions are employed.

In yet another aspect, the present invention provides a nucleic acid molecule comprising a sequence of nucleotides encoding a fusion polypeptide suitable for use in making a recombinant VLP, wherein said nucleic acid molecule encoded a POI and a particle associating portion of an L polypeptide and wherein the sequence of nucleotides encoding the particle associating portion of an L polypeptide comprises the sequence set forth in SEQ ID NO: 6 or SEQ ID NO: 8 or a contiguous sequence of nucleotides capable of hybridizing thereto or to a complementary form thereof under low stringency hybridisation conditions, or a functional derivative or homolog thereof.

Yet another aspect of the present invention provides a recombinant nucleic acid molecule for use in making a recombinant VLP, said nucleic acid molecule encoding a particle-associating portion of an L polypeptide of DHBV or a functional derivative or homolog thereof comprising and one or more cloning sites suitable for accepting a second nucleic acid molecule encoding one or more polypeptide of interest, wherein said polypeptides of interest is expressed as a fusion polypeptide with said L polypeptide.

Complementary forms of all or part the nucleic acid molecules of the present invention are expressly contemplated.

The terms "nucleic acids", "nucleotide" and "polynucleotide" include RNA, cDNA, genomic DNA, synthetic forms and mixed polymers, both sense and antisense strands, and may be chemically or biochemically modified or may contain non-natural or derivatized nucleotide bases, as will be readily appreciated by those skilled in the art. Such modifications include, for example, labels, methylation, substitution of one or more of the naturally occurring nucleotides with an analog (such as the morpholine ring), internucleotide modifications such as uncharged linkages (e.g. methyl phosphonates, phosphotriesters, phosphoamidates, carbamates, etc.), charged linkages (e.g. phosphorothioates, phosphorodithioates, etc.), pendent moieties (e.g. polypeptides), intercalators (e.g. acridine, psoralen, etc.), chelators, alkylators and modified linkages (e.g. α-anomeric nucleic acids, etc.). Also included are synthetic molecules that mimic polynucleotides in their ability to bind to a designated sequence *via* hydrogen binding and

other chemical interactions. Such molecules are known in the art and include, for example, those in which peptide linkages substitute for phosphate linkages in the backbone of the molecule.

The term "similarity" as used herein includes exact identity between compared sequences at the nucleotide or amino acid level. Where there is non-identity at the nucleotide level, "similarity" includes differences between sequences which result in different amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels.

Where there is non-identity at the amino acid level, "similarity" includes amino acids that are nevertheless related to each other at the structural, functional, biochemical and/or conformational levels. In a particularly preferred embodiment, nucleotide and sequence comparisons are made at the level of identity rather than similarity.

Terms used to describe sequence relationships between two or more polynucleotides or polypeptides include "reference sequence", "comparison window", "sequence similarity", "sequence identity", "percentage of sequence similarity", "percentage of sequence identity", "substantially similar" and "substantial identity". A "reference sequence" is at least 9 to 12 but frequently 15 to 18 and often at least 21 to 25 or above, such as 30 monomer units, inclusive of nucleotides and amino acid residues, in length. Because two polynucleotides may each comprise (1) a sequence (i.e. only a portion of the complete polynucleotide sequence) that is similar between the two polynucleotides, and (2) a sequence that is divergent between the two polynucleotides, sequence comparisons between two (or more) polynucleotides are typically performed by comparing sequences of the two polynucleotides over a "comparison window" to identify and compare local regions of sequence similarity. A "comparison window" refers to a conceptual segment of typically 12 contiguous residues that is compared to a reference sequence. The comparison window may comprise additions or deletions (i.e. gaps) of about 20% or less as compared to the reference sequence (which does not comprise additions or deletions) for optimal alignment of the two sequences. Optimal

alignment of sequences for aligning a comparison window may be conducted by computerised implementations of algorithms (GAP, BESTFIT, FASTA, and TFASTA in the Wisconsin Genetics Software Package Release 7.0, Genetics Computer Group, 575 Science Drive Madison, WI, USA) or by inspection and the best alignment (i.e. resulting in the highest percentage homology over the comparison window) generated by any of the various methods selected. Reference also may be made to the BLAST family of programs as, for example, disclosed by Altschul *et al*, Nucleic Acid Research, 25:3389-3402, 1997. A detailed discussion of sequence analysis can be found in Unit 19.3 of Ausubel et al *supra.*

The terms "sequence similarity" and "sequence identity" as used herein refers to the extent that sequences are identical or functionally or structurally similar on a nucleotide-by-nucleotide basis or an amino acid-by-amino acid basis over a window of comparison. Thus, a "percentage of sequence identity", for example, is calculated by comparing two optimally aligned sequences over the window of comparison, determining the number of positions at which the identical nucleic acid base (e.g. A, T, C, G, I) or the identical amino acid residue (e.g. Ala, Pro, Ser, Thr, Gly, Val, Leu, Ile, Phe, Tyr, Trp, Lys, Arg, His, Asp, Glu, Asn, Gln, Cys and Met) occurs in both sequences to yield the number of matched positions, dividing the number of matched positions by the total number of positions in the window of comparison (i.e., the window size), and multiplying the result by 100 to yield the percentage of sequence identity. For the purposes of the present invention, "sequence identity" will be understood to mean the "match percentage" calculated by the DNASIS computer program (Version 2.5 for windows; available from Hitachi Software engineering Co., Ltd., South San Francisco, California, USA) using standard defaults as used in the reference manual accompanying the software. Similar comments apply in relation to sequence similarity. Conservative amino acid changes may be considered to provide similar sequences but not identical sequences.

Particularly preferred nucleic acid molecules encoding a particle associating portion of an L polypeptide comprise a contiguous sequence of nucleotides substantially as set forth in SEQ ID NO: 6 or SEQ ID NO: 8 or a nucleotide sequence having at least about 50%

similarity to SEQ ID NO: 6 or SEQ ID NO: 8. Preferably the % similarity is in relation to the sequence encoding a particle associating portion of L polypeptide.

Furthermore, the present invention contemplates nucleic acid molecules encoding a particle associating portion of an L polypeptide comprising a sequence of nucleotides substantially as set forth in SEQ ID NO: 6 or SEQ ID NO: 8 or a nucleotide sequence capable of hybridizing to SEQ ID NO: 6 or SEQ ID NO: 8 or a complementary form thereof under medium stringency conditions.

Functional derivatives of the instant nucleic acid molecules include fragments thereof or sequences having one or more nucleotide mutations or modifications.

Mutations include one or more nucleotide deletions, insertions or substitutions. Alternatively or in addition, derivatives may be modified by the addition of sequences or moieties to enhance function such as enhanced stability or activity or to introduce new activity. For example, modifications may comprise the addition of fusagenic agents to enhance membrane permeability, modifications to affect pre or post-transcriptional modifications events, or to generate fusion proteins comprising labels, tags and other modifications for identification, purification and so forth.

Functional derivatives of the subject nucleic acid molecules retain the ability of the parent molecule to encode a polypeptide having the particle assembly function of S polypeptide or the particle associating function of L polypeptide.

Fragments of the nucleic acid molecules may include parts or one or more portions thereof, which have the function of the parent.

Functional homologs of the instant nucleic acid sequences include orthologus gene sequences from different species which are related by common phylogenic decent and also gene sequences from other species which are similar to the instant nucleic acid molecules as a result of convergent evolution, wherein the homologs are functionally and structurally

related to the instant nucleic acid sequences and are consequently readily identified and/or isolated by hybridization based methods or by sequence comparison with published genome databases. For example, the nucleotide sequence of approximately 20 avian hepadnaviruses are publicly available (Triyatni *et al*, J. Gen. Virol, *82*:373-378, 2001).

Similarity at the nucleic acid level may be assessed in assays exploiting different stringency of hybridization conditions as is well known in the art and is, for example, described in Ausubel *et al, supra.*

Reference herein to stringent hybridization conditions preferably means conditions which permit selective hybridization or annealing between molecules which are substantially similar. The hybridization temperature composition and ionic strength of the hybridization solution which meet this criteria will vary depending upon a number of well characterized factors such as length, degree of complementarity and GC content. For longer sequences it is generally possible to calculate the expected melting point of duplex nucleic acid sequences under various conditions. Hybridization may be to all or part of the instant polynucleotides with the minimum length being sufficient to provide specificity and functionality of their encoded polypeptides.

Low stringency hybridization conditions includes and encompasses from at least about 0 to at least about 15% v/v formamide and from at least about 1 M to at least about 2 M salt for hybridization, and at least about 1 M to at least about 2 M salt for washing conditions. Generally, low stringency is at from about 25-30°C to about 42°C. The temperature may be altered and higher temperatures used to replace formamide and/or to give alternative stringency conditions.

Medium stringency includes and encompasses from at least about 16% v/v to at least about 30% v/v formamide and from at least about 0.5 M to at least about 0.9 M salt for hybridization, and at least about 0.5 M to at least about 0.9 M salt for washing conditions. High stringency includes and encompasses from at least about 31% v/v to at least about 50% v/v formamide and from at least about 0.01 M to at least about 0.15 M salt for

hybridization, and at least about 0.01 M to at least about 0.15 M salt for washing conditions. In general, washing is carried out $T_m = 69.3 + 0.41$ (G+C%). However, the T_m of a duplex DNA decreases by 1°C with every increase of 1% in the number of mismatch base pairs (Bonner *et al*, 1974). Formamide is optional in these hybridization conditions. Accordingly, particularly preferred levels of stringency are defined as follows: low stringency is 6 x SSC buffer, 0.1% w/v SDS at 25-42°C; a moderate stringency is 2 x SSC buffer, 0.1% w/v SDS at a temperature in the range 20°C to 65°C; high stringency is 0.1 x SSC buffer, 0.1% w/v SDS at a temperature of at least 65°C.

The recombinant nucleic acid molecules of the present invention which are suitable for use in making recombinant VLPs may be introduced into vectors to facilitate VLP production.

Vectors, preferably contain cloning sites and are capable of autonomous replication in a defined host cell. Alternatively, the vector may integrate into the genome and replicate together with the chromosome into which it has been introduced. Vectors generally also include selection markers.

Examples of selectable markers include genes conferring resistance to compounds such as antibiotics, genes conferring the ability to grow on selected substrates, genes encoding proteins that produce detectable signals such as luminescence. A wide variety of such markers are known and available, including, for example, antibiotic resistance genes such as the neomycin resistance gene (*neo*) and the hygromycin resistance gene (*hyg*). Selectable markers also include genes conferring the ability to grow on certain media substrates such as the *tk* gene (thymidine kinase) or the *hprt* gene (hypoxanthine phosphoribosyltransferase) which confer the ability to grow on HAT medium (hypoxanthine, aminopterin and thymidine); and the bacterial *gpt* gene (guanine/xanthine phosphoribosyltransferase) which allows growth on MAX medium (mycophenolic acid, adenine and xanthine). Other selectable markers for use in mammalian cells and plasmids carrying a variety of selectable markers are described in Sambrook *et al., Molecular Cloning - A Laboratory Manual*, Cold Spring Harbour, New York, USA, 1990.

The selectable marker may depend on its own promoter for expression and the marker gene may not necessarily be derived from human genomes (e.g. prokaryotic marker genes may be used in human cells). However, it is preferable to replace the original promoter with transcriptional machinery known to function in the recipient cells. A large number of transcriptional initiation regions are available for such purposes including, for example, metallothionein promoters, thymidine kinase promoters, β-actin promoters, immunoglobulin promoters, SV40 promoters and human cytomegalovirus promoters. A widely used example is the pSV2-*neo* plasmid which has the bacterial neomycin phosphotransferase gene under control of the SV40 early promoter and confers in mammalian cells resistance to G418 (an antibiotic related to neomycin). A number of other variations may be employed to enhance expression of the selectable markers in animal cells, such as the addition of a poly(A) sequence and the addition of synthetic translation initiation sequences. Both constitutive and inducible promoters may be used.

The recombinant nucleic acid molecule for use in making a recombinant VLP are preferably in kit form to facilitate introduction of a nucleic acid molecule encoding a POI and particle assembly.

Another aspect of the present invention provides an expression vector comprising a sequence of nucleotides encoding a POI and at least a particle-associating portion of an L polypeptide of DHBV or a functional derivative or homolog thereof wherein said POI is capable of being expressed as a fusion polypeptide with said L polypeptide.

Another aspect of the present invention provides an expression vector comprising a sequence of nucleotides encoding a POI and at least a part of the S domain of an L polypeptide of DHBV or a functional derivative or homolog thereof wherein the POI is expressed within or at a location N terminal to the S-domain of the L polypeptide amino acid sequence or functional derivative of homolog thereof.

Preferably, the POI is expressed N terminally of the S domain of L polypeptide or within the pre-S domain of L polypeptide.

As will be understood by those skilled in the art, the nucleic acid molecules of the present invention may be further modified to ensure their suitability for expression in a range of cells, selection *in vitro*, suitability for cloning various POIs therein. Such techniques and strategies are well known to those skilled in the art and may be conveniently referred to in Ausbel *et al*, Eds short protocols in Molecular Biology, John Wiley and Sons, 5th Edition, 2002 and/or Sambrook *et al, supra*.

To ensure expression, the nucleotide sequences encoding the POI and the L polypeptide components are operatively linked to one or more expression control sequences. Preferably the two or more such nucleotide sequences are in the same reading frame.

In one embodiment expression vectors are conveniently stably integrated into the genome of host cells and expression is driven by host cell promoters.

The present invention also extends to microorganisms or host cells transformed or transfected or otherwise comprising a nucleic acid molecules comprising a sequence of nucleotides encoding a POI and at least a particle-associating portion of an L polypeptide of DHBV or a functional derivative or homolog thereof. Yeast cells are particularly convenient host cells. Prokaryotic or eukaryotic host cells are advantageously used. Typically prokaryotic cells include *E. coli*, *Bacillis sp* and eukaryotic cells include yeast, fungi, mammalian and insect cells.

The present invention furthermore provides a composition comprising a virus-like particle derived from DHBV L and S polypeptide for use as a vaccine wherein the L polypeptide comprises one or more antigens of interest.

The present invention also relates to a vaccine comprising an antigen of interest expressed as a fusion protein with a particle-associating L polypeptide of DHBV or a functional derivative or homolog thereof, and an S polypeptide of DHBV wherein the S polypeptide and antigen-L polypeptide are assembled into a VLP, in admixture with a suitable

pharmaceutically acceptable diluent or carrier. The vaccine may be lyophilized prior to use and may furthermore be admixed with suitable adjuvants. Accordingly the vaccine may be in kit form.

By "pharmaceutically acceptable" carrier, or diluent is meant a pharmaceutical vehicle comprised of a material that is not biologically or otherwise undesirable, i.e. the material may be administered to a subject along with the selected active agent without causing any or a substantial adverse reaction. Carriers may include excipients and other additives such as diluents, detergents, coloring agents, wetting or emusifying agents, pH buffering agents, preservatives, and the like.

The VLPs, and polypeptide nucleic acid molecules of the present invention can be formulated in pharmaceutical compositions which are prepared according to conventional pharmaceutical compounding techniques. See, for example, Remington's Pharmaceutical Sciences, 18th Ed. (1990, Mack Publishing, Company, Easton, PA, U.S.A.). The composition may contain the active agent or pharmaceutically acceptable salts of the active agent. These compositions may comprise, in addition to one of the active substances, a pharmaceutically acceptable excipient, carrier, buffer, stabilizer or other materials well known in the art. Such materials should be non-toxic and should not interfere with the efficacy of the active ingredient. The carrier may take a wide variety of forms depending on the form of preparation desired for administration, e.g. topical, intravenous, oral, intrathecal, epineural or parenteral.

For oral administration, the compounds can be formulated into solid or liquid preparations such as capsules, pills, tablets, lozenges, powders, suspensions or emulsions. In preparing the compositions in oral dosage form, any of the usual pharmaceutical media may be employed, such as, for example, water, glycols, oils, alcohols, flavoring agents, preservatives, coloring agents, suspending agents, and the like in the case of oral liquid preparations (such as, for example, suspensions, elixirs and solutions); or carriers such as starches, sugars, diluents, granulating agents, lubricants, binders, disintegrating agents and the like in the case of oral solid preparations (such as, for example, powders, capsules and

tablets). Because of their ease in administration, tablets and capsules represent the most advantageous oral dosage unit form, in which case solid pharmaceutical carriers are obviously employed. If desired, tablets may be sugar-coated or enteric-coated by standard techniques. The active agent can be encapsulated to make it stable to passage through the gastrointestinal tract while at the same time allowing for passage across the blood brain barrier. See for example, International Patent Publication No. WO 96/11698.

For parenteral administration, the compound may be dissolved in a pharmaceutical carrier and administered as either a solution of a suspension. Illustrative of suitable carriers are water, saline, dextrose solutions, fructose solutions, ethanol, or oils of animal, vegetative or synthetic origin. The carrier may also contain other ingredients, for example, preservatives, suspending agents, solubilizing agents, buffers and the like. When the compounds are being administered intrathecally, they may also be dissolved in cerebrospinal fluid.

The active agent is preferably administered in a therapeutically effective amount. The actual amount administered and the rate and time-course of administration will depend on the nature and severity of the condition being treated. Prescription of treatment, e.g. decisions on dosage, timing, etc. is within the responsibility of general practitioners or specialists and typically takes account of the disorder to be treated, the condition of the individual patient, the site of delivery, the method of administration and other factors known to practitioners. Examples of techniques and protocols can be found in Remington's Pharmaceutical Sciences, *supra*.

Alternatively, targeting therapies may be used to deliver the active agent more specifically to certain types of cell, by the use of targeting systems such as antibodies or cell specific ligands or specific nucleic acid molecules. Targeting may be desirable for a variety of reasons, e.g. if the agent is self-antigenic or if it would not otherwise be able to enter the target cells.

Instead of administering these agents directly, they could be produced in the target cell, e.g. in a viral vector such as described below or in a cell based delivery system such as described in U.S. Patent No. 5,550,050 and International Patent Publication Nos. WO 92/19195, WO 94/25503, WO 95/01203, WO 95/05452, WO 96/02286, WO 96/02646, WO 96/40871, WO 96/40959 and WO 97/12635. The vector could be targeted to the target cells. The cell based delivery system is designed to be implanted in a patient's body at the desired target site and contains a coding sequence for the target agent. Alternatively, the agent could be administered in a precursor form for conversion to the active form by an activating agent produced in, or targeted to, the cells to be treated. See, for example, European Patent Application No. 0 425 731A and International Patent Publication No. WO 90/07936.

Vaccine composition may alternatively comprise nucleic acid molecules encoding the recombinant VLPs.

Gene transfer systems known in the art may be useful in the practice of genetic manipulation. These include viral and non-viral transfer methods. A number of viruses have been used as gene transfer vectors or as the basis for preparing gene transfer vectors, including papovaviruses (e.g. SV40, Madzak *et al.*, *J. Gen. Virol. 73:* 1533-1536, 1992), adenovirus (Berkner, *Curr. Top. Microbiol. Immunol. 158:* 39-66, 1992; Berkner *et al.*, *BioTechniques 6;* 616-629, 1988; Gorziglia and Kapikian, *J. Virol. 66:* 4407-4412, 1992; Quantin *et al.*, *Proc. Natl. Acad. Sci. USA 89:* 2581-2584, 1992; Rosenfeld *et al.*, *Cell 68:* 143-155, 1992; Wilkinson *et al.*, *Nucleic Acids Res. 20:* 2233-2239, 1992; Stratford-Perricaudet *et al.*, *Hum. Gene Ther. 1:* 241-256, 1990; Schneider *et al.*, *Nature Genetics 18:* 180-183, 1998), vaccinia virus (Moss, *Curr. Top. Microbiol. Immunol. 158:* 25-38, 1992; Moss, *Proc. Natl. Acad. Sci. USA 93:* 11341-11348, 1996), adeno-associated virus (Muzyczka, *Curr. Top. Microbiol. Immunol. 158:* 97-129, 1992; Ohi *et al.*, *Gene 89:* 279-282, 1990; Russell and Hirata, *Nature Genetics 18:* 323-328, 1998), herpesviruses including HSV and EBV (Margolskee, *Curr. Top., Microbiol. Immunol. 158:* 67-95, 1992; Johnson *et al.*, *J. Virol. 66:* 2952-2965, 1992; Fink *et al.*, *Hum. Gene Ther. 3:* 11-19, 1992; Breakefield and Geller, *Mol. Neurobiol. 1:* 339-371, 1987; Freese *et al.*, *Biochem.*

Pharmacol. 40: 2189-2199, 1990; Fink *et al.*, *Ann. Rev. Neurosci. 19:* 265-287, 1996), lentiviruses (Naldini *et al.*, *Science 272:* 263-267, 1996), Sindbis and Semliki Forest virus (Berglund *et al.*, *Biotechnology 11:* 916-920, 1993) and retroviruses of avian (Bandyopadhyay and Temin, *Mol. Cell. Biol. 4:* 749-754, 1984; Petropoulos *et al.*, *J. Viol. 66:* 3391-3397, 1992], murine [Miller, *Curr. Top. Microbiol. Immunol. 158:* 1-24, 1992; Miller *et al.*, *Mol. Cell. Biol. 5:* 431-437, 1985; Sorge *et al.*, *Mol. Cell. Biol. 4:* 1730-1737, 1984; Mann and Baltimore, *J. Virol. 54:* 401-407, 1985; Miller *et al.*, *J. Virol. 62:* 4337-4345, 1988] and human [Shimada *et al.*, *J. Clin. Invest. 88:* 1043-1047, 1991; Helseth *et al.*, *J. Virol. 64:* 2416-2420, 1990; Page *et al.*, *J. Virol. 64:* 5270-5276, 1990; Buchschacher and Panganiban, *J. Virol. 66:* 2731-2739, 1982] origin.

Non-viral gene transfer methods are known in the art such as chemical techniques including calcium phosphate co-precipitation, mechanical techniques, for example, microinjection, membrane fusion-mediated transfer *via* liposomes and direct DNA uptake and receptor-mediated DNA transfer. Viral-mediated gene transfer can be combined with direct *in vivo* gene transfer using liposome delivery, allowing one to direct the viralvectors to particular cells. Alternatively, the retroviral vector producer cell line can be injected into particular tissue. Injection of producer cells would then provide a continuous source of vector particles.

In an approach which combines biological and physical gene transfer methods, plasmid DNA of any size is combined with a polylysine-conjugated antibody specific to the adenovirus hexon protein and the resulting complex is bound to an adenovirus vector. The trimolecular complex is then used to infect cells. The adenovirus vector permits efficient binding, internalization and degradation of the endosome before the coupled DNA is damaged. For other techniques for the delivery of adenovirus based vectors, see U.S. Patent No. 5,691,198.

Liposome/DNA complexes have been shown to be capable of mediating direct *in vivo* gene transfer. While in standard liposome preparations the gene transfer process is non-specific,

localized *in vivo* uptake and expression have been reported in tumor deposits, for example, following direct *in situ* administration.

General methods for generating the viral particles of the present invention are well know to skilled practitioners.

The present invention provides a method of preparing a recombinant VLP said method comprising:

i) cloning a nucleic acid molecule encoding a polypeptide of interest into an expression vector comprising a particle-associating portion of an L polypeptide of DHBV or a functional derivative or homolog thereof;

ii) infecting or transfecting a suitable cell with the recombinant expression vector of step i) under conditions which allow protein expression and particle assembly with S polypeptide of DHBV or a functional derivative or homolog thereof;

iii) recovering said virus-like particles from said cells.

Yeast cells, for example, will be transformed with L POI and S expression plasmids. Preferably, expression is driven by a yeast promoter and expressed in yeast cells, such as a strain of *Saccharomyces*.

Another aspect of the present invention is directed to antibodies to the fusion polypeptides of the present invention. Antibodies may be monoclonal or polyclonal and technique for their manufacture are very well known. Antibodies which specifically recognise determinants of the fusion polypeptide of the present invention are particularly preferred.

The present invention is further described by the further non-limiting Examples.

EXAMPLE 1

Substitution of a.a.45-125 of DHBV preS with the ectodomain of HCV E2 by Fusion PCR

Fusion PCR (overlap extension PCR) was used as described by Ho *et al*, Gene, 77:51, 1989. Overlapping primers were each paired with an outside primer complementary to the plus strand of pCDL-w.t. or the minus strand of HCV construct in 2 first round PCR reactions using pfu enzyme. The PCR products (153 bp from pCDL as template and 272 bp with the HCV template) from each reaction were purified using a Qiagen min elute kit and the two purified products used as the template for the fusion PCR reaction using the outside primers. The 578 bp fusion PCR product was purified and digested with Xma1, cutting at nt 1743 of the HCV primer sequence and with Aat II, cutting at nt 831 of DHBV L. The plasmid, pMDL-w.t., carrying unique Xma1 and Aat II sites in the DHBV preS coding sequence was used as vector. The digested PCR fragment and the large fragment of the cut vector were excised from an agarose gel and purified using a Prep-a-gene kit (Bio-Rad). Competent cells (DH5 cells) were transformed with the ligated plasmid and transformants selected from ampicillin plates.

Positive clones were detected by restriction enzyme digestion of purified DNA using a restriction site which is also present in the HCV E2 ectodomain sequence (Bsa1).

A Sal1/Xho 1 fragment of pMDL-E2 containing the E2 insert was subcloned into pCDL-w.t. (DHBV L expression plasmid with CMV promotor) using the same unique restriction sites. Bsa 1 digestion was used again to confirm the presence of the E2 insert in pCDL-E2. The CDL-E2 clone was also confirmed by sequencing, covering the region of preS-E2-preS and part of S to nucleotide 1581.

Outside Primers

P804
5' GGGCAACATCCAGCAAAATCAATGG 3' (SEQ ID NO: 1 DHBV nt 804-828)

P-1719
5' GCTGCGGAATGGCTAAAAGGGCCCCCGACC 3' (SEQ ID NO: 2 HCV nt 1719-1749 with an Xma1 RE site inserted, shown underlined)

Overlapping Chimeric Primers (plain type= DHBVpreS; **bold**= HCV E2)

P1492 (refers to nt at start of E2 sequence)
CCAACACTAGATCAC**GAAACCCACGTCACCGGGG** (SEQ ID NO: 3)

P-1492
GGTTGTGATCTAGTG**CTTTGGGTGCAGTGGCCCC** (SEQ ID NO: 4)

Templates:
pCDL-wt (DHBV L expression plasmid); p90/HCV FL-longpU

EXAMPLE 2

Expression and Analysis of CDL-E2 in avian Hepatoma (LMH) cells

The avian hepatoma cell line, LMH was co-transfected with 5 μg each of pCDL-E2 and pCI-S (Gazina *et al*, Virology *242*:266, 1998) using the dextran sulphate method (Grgacic *et al*, J.Gen. Virol. *79*:2743, 1998). Day 3 post-transfection media were collected for assessment of exported particles and cells either processed for cytosolic fractionation and assessment of intracellular particle formation or isolation of microsomes for protease protection analysis (Grgacic, J.Gen.Virol. *83*:1635, 2002).

EXAMPLE 3

Isolation of Intracellular and Extracellular Particles

(i) Extracellular particles: Media from transfected LMH cells was harvested day 3 post-transfection and clarified of non-adherent cells by centrifugation for 5 min at 2,000 rpm.

(ii) Intracellular particles: Cell monolayers were washed twice with PBS and harvested by scaping cells into 1 ml PBS. Harvested cells were freeze/thawed three times with vigorous vortexing upon thawing. The cytosol fraction (supernatant) was obtained by centrifugation for 1 min at 10,000 rpm in an Eppendorf centrifuge. This procedure has been used in this laboratory to release DHBV particles from transfected cells capable of infecting primary duck hepatocytes.

Particles in the clarified media or cytosol fraction were diluted to 6 ml with PBS and pelleted for 3 h at 38,000 rpm in an SW40 rotor (Beckman) through 3 ml of 20% sucrose onto a 2 ml 70% sucrose cushion. The fraction at the 20-70% interface was collected from the bottom, methanal precipitated for 16 h at -20°C followed by separation on 13% SDS-PAGE and Western blotting.

EXAMPLE 4

Protease Protection Analysis

Microsomes were prepared according to the method of Prange and Streeck, EMBO J. *14*:247, 1995 with modifications. Transfected LMH cells (two 30mm diameter wells) were washed in cold Tris-buffered saline (TBS: 50mM Tris-HCl, pH 7.5; 150mM NaCl).

The monolayers in each well were incubated on ice with 0.4 ml 0.1X TBS for 10 minutes and then harvested by scraping, pooled and dispersed by drawing 5 times through a 26G needle. The homogenate was adjusted to 1X TBS with 5X TBS and centrifuged for 20 min at 2,500 rpm at 4°C to remove unbroken cells and nuclei. The supernatant was removed and set aside while the pellet was again dispersed in 300µl TBS and centrifuged as before.

Supernatants were pooled and layered onto 2.7ml 250mM sucrose in TBS and centrifuged for 30min. at 38,000rpm at 4°C in an SW-60 rotor (Beckman). The microsomal pellets were washed once with TBS and resuspended in 65µl TBS.

For trypsin protection analysis the microsomal preparation was divided into three 20 µl aliquots. One sample was left untreated while the remaining two were treated with 25µg/ml of trypsin (TPCK treated; Worthington Biochem. Corp. NJ. USA) with or without 0.5% NP-40 for 1 h. on ice.

EXAMPLE 5

Western blot analysis

Proteins were separated by SDS-PAGE (13% acrylamide) and transferred to nitrocellulose membrane(Schleicher and Schüll) using a Trans-Blot SD semi-dry transfer cell (Biorad). Membranes were blocked for 1 h with 3% skim milk in PBS plus 0.3% Tween 20 (PBST). Membranes were probed with monoclonal anti-S (7C.12) (Pugh *et al*, J. Virol. *69*:4814, 1995) for 1 h in 1% skim milk; PBST, then washed with PBST and probed with goat anti-mouse Ig:horse radish peroxidase (Amersham) in 1% skim milk PBST. After a final wash in PBST (3x10 min.) proteins bands were visualised by enhanced chemiluminescence (ECL) (Amersham).

EXAMPLE 6

Construction of strategically selected chimeric DHBV VLPs to define their carrying capacity as a potential vaccine delivery vehicle

The receptor binding region as well as the C terminus of preS is exposed to the DHBV subviral particle surface. These exposed regions, flanked by the membrane spanning S domain, are believed to be further stabilised through anchorage at the N terminus by the myristylation signal. The HCV E2 ectodomain inserted into this region of preS was similarly exposed and stabilised. PreS sequences are substituted by equivalent or larger

sized foreign sequences or alternatively fused in frame to the N terminus of the S domain of L by fusion PCR.

To aid translocation of the chimeric L polypeptides, an L construct with a signal sequence such as the preprolactin signal sequence fused to the N terminus, which causes co-translational translocation of L, is also used. These SigL chains can assemble with S subunits and be exported as particles . Translocation of the chimeric preS domains is monitored by the protease protection assay and antibody mapping of the topology on the assembled particle by immunoprecipitation. Particles will be purified by sucrose gradient sedimentation and analysed by EM/imunogold labelling for VLP formation. Pulse-chase metabolic labelling will be performed to assess that the proportion of recombinant chains relative to S (approximately 1:4 for wild type DHBV) is maintained in the assembled particle.

Those skilled in the art will appreciate that the invention described herein is susceptible to variations and modifications other than those specifically described. It is to be understood that the invention includes all such variations and modifications. The invention also includes all of the steps, features, compositions and compounds referred to or indicated in this specification, individually or collectively, and any and all combinations of any two or more of said steps or features.

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        35                  40                  45

cat gtg caa aca atg gag gag ata aac acc ctc cag aat cag gga gct      192
His Val Gln Thr Met Glu Glu Ile Asn Thr Leu Gln Asn Gln Gly Ala
    50                  55                  60

tgg cct gct ggg gcg gga agg aga gta gga tta tca aat ccg act cct      240
Trp Pro Ala Gly Ala Gly Arg Arg Val Gly Leu Ser Asn Pro Thr Pro
65                  70                  75                  80

caa gag att cct cag ccc cag tgg act ccc gag gaa gac caa aaa gca      288
Gln Glu Ile Pro Gln Pro Gln Trp Thr Pro Glu Glu Asp Gln Lys Ala
                85                  90                  95

cgc gaa gct ttt cgc cgt tat caa gaa gaa aga cca ccg gaa acc acc      336
Arg Glu Ala Phe Arg Arg Tyr Gln Glu Glu Arg Pro Pro Glu Thr Thr
            100                 105                 110

acc att cct ccg tct tcc cct cct cag tgg aag cta caa ccc ggg gac      384
Thr Ile Pro Pro Ser Ser Pro Pro Gln Trp Lys Leu Gln Pro Gly Asp
        115                 120                 125

gat cca ctc ctg gga aat cag tct ctc ctc gag act cat ccg cta tac      432
Asp Pro Leu Leu Gly Asn Gln Ser Leu Leu Glu Thr His Pro Leu Tyr
    130                 135                 140
```

cag tca gaa cca gcg gtg cca gtg ata aaa act ccc ccc ttg aag aag 480
Gln Ser Glu Pro Ala Val Pro Val Ile Lys Thr Pro Pro Leu Lys Lys
145 150 155 160

aaa 483
Lys

<210> 11

<211> 161

<212> PRT

<213> duck

<400> 11

Met Gly Gln His Pro Ala Lys Ser Met Asp Val Arg Arg Ile Glu Gly
1 5 10 15

Gly Glu Ile Leu Leu Asn Gln Leu Ala Gly Arg Met Ile Pro Lys Gly
20 25 30

Thr Leu Thr Trp Ser Gly Lys Phe Pro Thr Leu Asp His Val Leu Asp
35 40 45

His Val Gln Thr Met Glu Glu Ile Asn Thr Leu Gln Asn Gln Gly Ala
50 55 60

Trp Pro Ala Gly Ala Gly Arg Arg Val Gly Leu Ser Asn Pro Thr Pro
65 70 75 80

Gln Glu Ile Pro Gln Pro Gln Trp Thr Pro Glu Glu Asp Gln Lys Ala
85 90 95

Arg Glu Ala Phe Arg Arg Tyr Gln Glu Glu Arg Pro Pro Glu Thr Thr
100 105 110

Thr Ile Pro Pro Ser Ser Pro Pro Gln Trp Lys Leu Gln Pro Gly Asp
115 120 125

Asp Pro Leu Leu Gly Asn Gln Ser Leu Leu Glu Thr His Pro Leu Tyr
130 135 140

Gln Ser Glu Pro Ala Val Pro Val Ile Lys Thr Pro Pro Leu Lys Lys
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Lys

<210> 12

<211> 501

<212> DNA

<213> duck

<220>

<221> CDS

<222> (1)..(501)

<400> 12

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Met Ser Gly Thr Phe Gly Gly Ile Leu Ala Gly Leu Ile Gly Leu Leu
1               5                  10                  15

gta agc ttt ttc ttg ttg ata aaa att cta gaa ata ctg agg agg cta      96
Val Ser Phe Phe Leu Leu Ile Lys Ile Leu Glu Ile Leu Arg Arg Leu
            20                  25                  30

gat tgg tgg tgg att tct ctc agt tct cca aag gga aaa atg caa tgc     144
Asp Trp Trp Trp Ile Ser Leu Ser Ser Pro Lys Gly Lys Met Gln Cys
        35                  40                  45

gct ttc caa gat act gga gcc caa atc tct cca cat tac gta gga tct     192
Ala Phe Gln Asp Thr Gly Ala Gln Ile Ser Pro His Tyr Val Gly Ser
    50                  55                  60

tgc ccg tgg gga tgc cca gga ttt ctt tgg acc tat ctc agg ctt ttt     240
Cys Pro Trp Gly Cys Pro Gly Phe Leu Trp Thr Tyr Leu Arg Leu Phe
65                  70                  75                  80

atc atc ttc ctc tta atc ctg cta gta gca gca ggc ttg ctg tat ctg     288
Ile Ile Phe Leu Leu Ile Leu Leu Val Ala Ala Gly Leu Leu Tyr Leu
                85                  90                  95

acg gac aac ggg tct act att tta gga aag ctc caa tgg gcg tcg gtc     336
Thr Asp Asn Gly Ser Thr Ile Leu Gly Lys Leu Gln Trp Ala Ser Val
            100                 105                 110

tca gcc ctt ttc tcc tcc atc tct tca cta ctg ccc tcg gat ccg aaa     384
Ser Ala Leu Phe Ser Ser Ile Ser Ser Leu Leu Pro Ser Asp Pro Lys
```

```
        115                 120                 125

tct ctc gtc gct tta acg ttt gga ctt tca ctt ata tgg atg act tcc      432
Ser Leu Val Ala Leu Thr Phe Gly Leu Ser Leu Ile Trp Met Thr Ser
    130                 135                 140

tcc tct gcc acc caa acg ctc gtc acc tta acg caa tta gcc acg ctg      480
Ser Ser Ala Thr Gln Thr Leu Val Thr Leu Thr Gln Leu Ala Thr Leu
145                 150                 155                 160

tct gct ctt ttt tac aag agt                                          501
Ser Ala Leu Phe Tyr Lys Ser
                165
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<210> 13

<211> 167

<212> PRT

<213> duck

<400> 13

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Met Ser Gly Thr Phe Gly Gly Ile Leu Ala Gly Leu Ile Gly Leu Leu
1               5                   10                  15

Val Ser Phe Phe Leu Leu Ile Lys Ile Leu Glu Ile Leu Arg Arg Leu
            20                  25                  30

Asp Trp Trp Trp Ile Ser Leu Ser Ser Pro Lys Gly Lys Met Gln Cys
        35                  40                  45

Ala Phe Gln Asp Thr Gly Ala Gln Ile Ser Pro His Tyr Val Gly Ser
    50                  55                  60

Cys Pro Trp Gly Cys Pro Gly Phe Leu Trp Thr Tyr Leu Arg Leu Phe
65                  70                  75                  80

Ile Ile Phe Leu Leu Ile Leu Leu Val Ala Ala Gly Leu Leu Tyr Leu
                85                  90                  95

Thr Asp Asn Gly Ser Thr Ile Leu Gly Lys Leu Gln Trp Ala Ser Val
            100                 105                 110

Ser Ala Leu Phe Ser Ser Ile Ser Ser Leu Leu Pro Ser Asp Pro Lys
        115                 120                 125
```

Ser Leu Val Ala Leu Thr Phe Gly Leu Ser Leu Ile Trp Met Thr Ser
130 135 140

Ser Ser Ala Thr Gln Thr Leu Val Thr Leu Thr Gln Leu Ala Thr Leu
145 150 155 160

Ser Ala Leu Phe Tyr Lys Ser
165

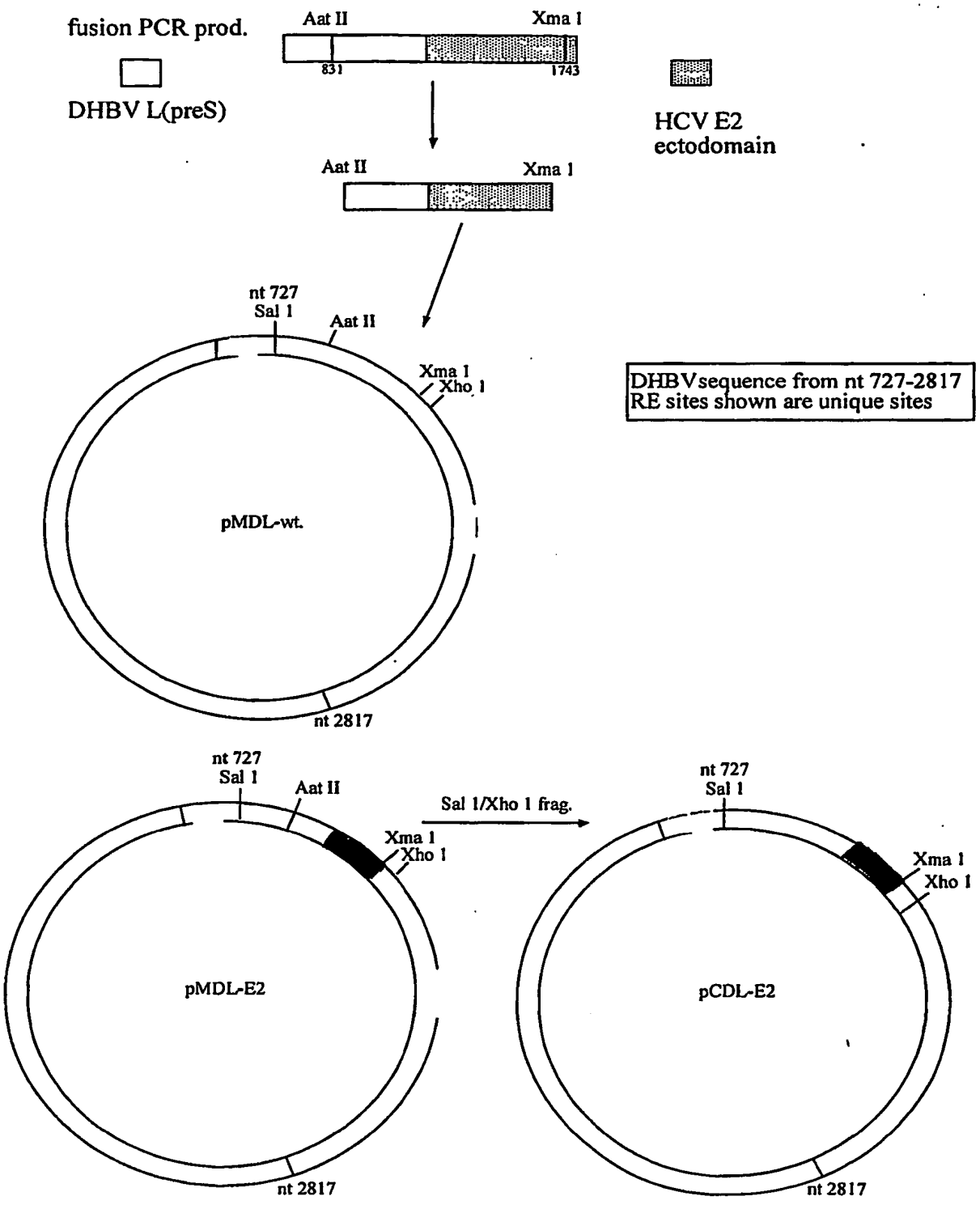
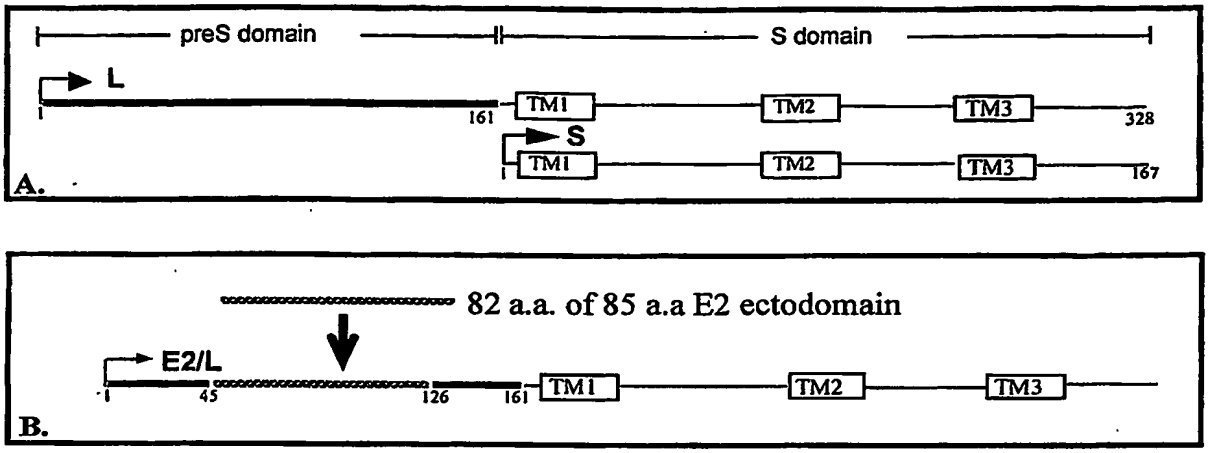

fusion PCR prod.
DHBV L(preS)
Aat II
Xma I
831
1743
HCV E2
ectodomain
Aat II
Xma I
nt 727
Sal I
Aat II
Xma I
Xho I
pMDL-wt.
nt 2817
DHBV sequence from nt 727-2817
RE sites shown are unique sites
nt 727
Sal I
Aat II
Xma I
Xho I
pMDL-E2
nt 2817
Sal I/Xho I frag.
nt 727
Sal I
Xma I
Xho I
pCDL-E2
nt 2817

FIGURE 1



C. E2/L chimera is translocated across the ER membrane

trypsin	-	+	+
NP-40	-	-	+

E2/L—

S —

microsomes:protease protection assay

D. E2/L chimera is assembled into particles

E2/L—

S —

particles purified by sedimentation through 20% sucrose

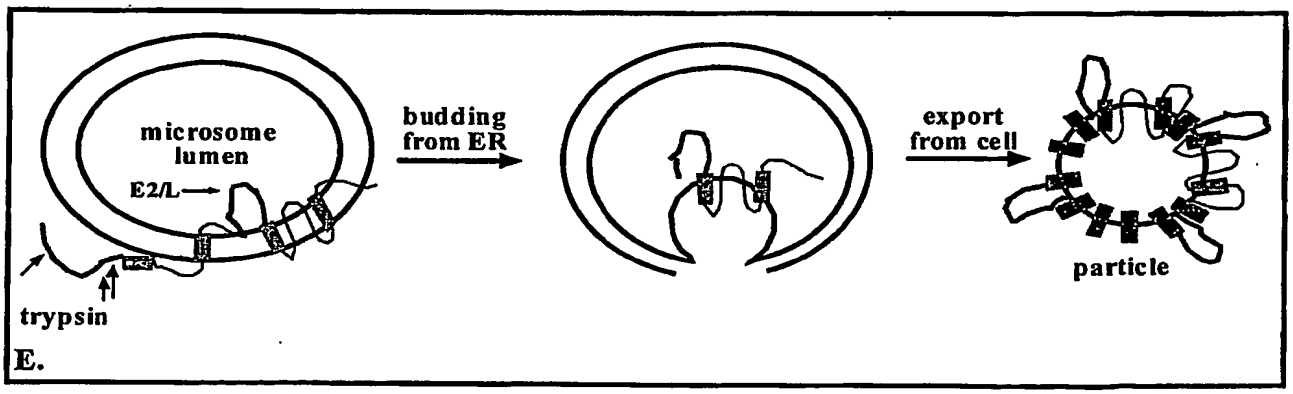


FIGURE 2

DHBV full genome sequence (US D16 Acc. No. K01839)

1 catgctcatt tgaaagctta tgcaaaaatt aacgaggaat cactggatag ggctaggaga
61 ttgctttggt ggcattacaa ctgtttactg tggggagaag ctcaagttac taactatatt
121 tctcgtttgc gtacttggtt gtcaactcct gagaaatata gaggtagaga tgccccgacc
181 attgaagcaa tcactagacc aatccaggtg gctcagggag gcagaaaaac aactacgggt
241 actagaaaac ctcgtggact cgaacctaga agaagaaaag ttaaaaccac agttgtctat
301 gggagaagac gttcaaagtc ccgggaaagg agagccccta caccccaacg tgcgggctcc
361 cctctcccac gtagttcgag cagccaccat agatctccct cgcctaggaa ataaattacc
421 tgctaggcat cacttaggta aattgtcagg actatatcaa atgaagggct gtacttttaa
481 cccagaatgg aaagtaccag atatttcgga tactcatttt aatttagatg tagttaatga
541 gtgcccttcc cgaaattgga aatatttgac tccagccaaa ttctggccca agagcatttc
601 ctactttcct gtccaggtag gggttaaacc aaagtatcct gacaatgtga tgcaacatga
661 atcaatagta ggtaaatatt taaccaggct ctatgaagca ggaatccttt ataagcggat
721 atctaaacat ttggtcacat ttaaaggtca gccttataat tgggaacagc aacaccttgt
781 caatcaacat cacatttatg atggggcaac atccagcaaa atcaatggac gtcagacgga
841 tagaaggagg agaaatactg ttaaaccaac ttgccggaag gatgatccca aaagggactt
901 tgacatggtc aggcaagttt ccaacactag atcacgtgtt agaccatgtg caaacaatgg
961 aggagataaa caccctccag aatcagggag cttggcctgc tggggcggga aggagagtag
1021 gattatcaaa tccgactcct caagagattc ctcagcccca gtggactccc gaggaagacc
1081 aaaaagcacg cgaagctttt cgccgttatc aagaagaaag accaccggaa accaccacca
1141 ttcctccgtc ttcccctcct cagtggaagc tacaacccgg ggacgatcca ctcctgggaa
1201 atcagtctct cctcgagact catccgctat accagtcaga accagcggtg ccagtgataa
1261 aaactccccc cttgaagaag aaaatgtctg gtaccttcgg gggaatacta gctggcctaa
1321 tcggattact ggtaagcttt ttcttgttga taaaaattct agaaatactg aggaggctag
1381 attggtggtg gatttctctc agttctccaa agggaaaaat gcaatgcgct ttccaagata
1441 ctggagccca aatctctcca cattacgtag gatcttgccc gtggggatgc ccaggatttc
1501 tttggaccta tctcaggctt tttatcatct tcctcttaat cctgctagta gcagcaggct
1561 tgctgtatct gacggacaac gggtctacta ttttaggaaa gctccaatgg gcgtcggtct
1621 cagccctttt ctcctccatc tcttcactac tgccctcgga tccgaaatct ctcgtcgctt
1681 taacgtttgg actttcactt atatggatga cttcctcctc tgccacccaa acgctcgtca
1741 ccttaacgca attagccacg ctgtctgctc ttttttacaa gagttaggaa taagaataaa
1801 ctttgacaaa accacgcctt ctccggtgaa tgaaataaga ttcctcggtt accagattga
1861 tgaaaatttc atgaagattg aagaaagcag atggaaagaa ttaaggactg taatcaagaa
1921 aataaaagta ggagaatggt atgactggaa atgtattcaa agatttgtgg ggcatttgaa
1981 ttttgttttg ccttttacta aaggtaatat tgaaatgtta aaaccaatgt atgctgctat
2041 tactaaccaa gtaaacttta gcttctcttc atcctatagg actttgttat ataaactaac
2101 aatgggtgtg tgtaaattaa gaataaagcc aaagtcctct gtacctttgc cacgtgtagc

FIGURE 3

2161 tacagatgct accccaacac atggcgcaat atcccatatc accggcggga gcgcagtgtt
2221 tgctttttca aaggtcagag atatacatgt tcaggaacta ttgatgtctt gtttagccaa
2281 gataatgatt aaaccacgtt gtctcttatc tgattcaact tttgtttgcc ataagcgtta
2341 tcagacgtta ccatggcatt ttgctatgtt ggccaaacaa ttgctcaaac cgatacaatt
2401 gtactttgtc ccgagcaaat ataatcctgc tgacggccca tccaggcaca aacctcctga
2461 ttggacggct tttccataca cccctctctc gaaagcaata tatattccac ataggctatg
2521 tggaacttaa gaattacacc cctctccttc ggagctgctt gccaaggtat ctttacgtct
2581 acattgctgt tgtcgtgtgt gactgtacct ttggtatgta ccattgttta tgattcttgc
2641 ttatatatgg atatcaatgc ttctagagcc ttagccaatg tgtatgatct accagatgat
2701 ttctttccaa aaatagatga tcttgttaga gatgctaaag acgctttaga gccttattgg
2761 aaatcagatt caataaagaa acatgttttg attgcaactc actttgtgga tctcattgaa
2821 gacttctggc agactacaca gggcatgcat gaaatagccg aatcattaag agctgttata
2881 cctcccacta ctactcctgt tccaccgggt tatcttattc agcacgagga agctgaagag
2941 ataccttttgg gagatttatt taaacaccaa gaagaaagga tagtaagttt ccaacccgac
3001 tatccgatta cggctagaat t

FIGURE 3 Cont.

DHBV L sequence (US D16) (start L atg 801; start S atg 1284)

```
801/1                                                   831/11
atg ggg caa cat cca gca aaa tca atg gac gtc aga cgg ata gaa gga gga gaa ata ctg
M   G   Q   H   P   A   K   S   M   D   V   R   R   I   E   G   G   E   I   L
861/21                                                  891/31
tta aac caa ctt gcc gga agg atg atc cca aaa ggg act ttg aca tgg tca ggc aag ttt
L   N   Q   L   A   G   R   M   I   P   K   G   T   L   T   W   S   G   K   F
921/41                                                  951/51
cca aca cta gat cac gtg tta gac cat gtg caa aca atg gag gag ata aac acc ctc cag
P   T   L   D   H   V   L   D   H   V   Q   T   M   E   E   I   N   T   L   Q
981/61                                                  1011/71
aat cag gga gct tgg cct gct ggg gcg gga agg aga gta gga tta tca aat ccg act cct
N   Q   G   A   W   P   A   G   A   G   R   R   V   G   L   S   N   P   T   P
1041/81                                                 1071/91
caa gag att cct cag ccc cag tgg act ccc gag gaa gac caa aaa gca cgc gaa gct ttt
Q   E   I   P   Q   P   Q   W   T   P   E   E   D   Q   K   A   R   E   A   F
1101/101                                                1131/111
cgc cgt tat caa gaa gaa aga cca ccg gaa acc acc acc att cct ccg tct tcc cct cct
R   R   Y   Q   E   E   R   P   P   E   T   T   T   I   P   P   S   S   P   P
1161/121                                                1191/131
cag tgg aag cta caa ccc ggg gac gat cca ctc ctg gga aat cag tct ctc ctc gag act
Q   W   K   L   Q   P   G   D   D   P   L   L   G   N   Q   S   L   L   E   T
1221/141                                                1251/151
cat ccg cta tac cag tca gaa cca gcg gtg cca gtg ata aaa act ccc ccc ttg aag aag
H   P   L   Y   Q   S   E   P   A   V   P   V   I   K   T   P   P   L   K   K
1281/161                                                1311/171
```

FIGURE 4

```
aaa atg tct ggt acc ttc ggg gga ata cta gct ggc cta atc gga tta ctg gta agc ttt
K   M   S   G   T   F   G   G   I   L   A   G   L   I   G   L   L   V   S   F
1341/181                                1371/191
ttc ttg ttg ata aaa att cta gaa ata ctg agg agg cta gat tgg tgg tgg att tct ctc
F   L   L   I   K   I   L   E   I   L   R   R   L   D   W   W   W   I   S   L
1401/201                                1431/211
agt tct cca aag gga aaa atg caa tgc gct ttc caa gat act gga gcc caa atc tct cca
S   S   P   K   G   K   M   Q   C   A   F   Q   D   T   G   A   Q   I   S   P
1461/221                                1491/231
cat tac gta gga tct tgc ccg tgg gga tgc cca gga ttt ctt tgg acc tat ctc agg ctt
H   Y   V   G   S   C   P   W   G   C   P   G   F   L   W   T   Y   L   R   L
1521/241                                1551/251
ttt atc atc ttc ctc tta atc ctg cta gta gca gca ggc ttg ctg tat ctg acg gac aac
F   I   I   F   L   L   I   L   L   V   A   A   G   L   L   Y   L   T   D   N
1581/261                                1611/271
ggg tct act att tta gga aag ctc caa tgg gcg tcg gtc tca gcc ctt ttc tcc tcc atc
G   S   T   I   L   G   K   L   Q   W   A   S   V   S   A   L   F   S   S   I
1641/281                                1671/291
tct tca cta ctg ccc tcg gat ccg aaa tct ctc gtc gct tta acg ttt gga ctt tca ctt
S   S   L   L   P   S   D   P   K   S   L   V   A   L   T   F   G   L   S   L
1701/301                                1731/311
ata tgg atg act tcc tcc tct gcc acc caa acg ctc gtc acc tta acg caa tta gcc acg
I   W   M   T   S   S   S   A   T   Q   T   L   V   T   L   T   Q   L   A   T
1761/321                                1791/331
ctg tct gct ctt ttt tac aag agt tag
L   S   A   L   F   Y   K   S   *
```

FIGURE 4 Cont.